PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference P10391 PC/P10387	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/EP 02/14895	International filing date *(day/month/year)* 27.12.2002	Priority date *(day/month/year)* 28.12.2001
International Patent Classification (IPC) or both national classification and IPC C12N5/08		
Applicant CELL THERAPEUTICS SCANDINAVIA AB et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 8 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02.07.2003	01.04.2004
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized Officer Mandl, B Telephone No. +49 89 2399-8434

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP 02/14895

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, Pages

1-20 as originally filed

Claims, Numbers

1-56 received on 14.01.2004 with letter of 14.01.2004

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readable form.

   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application,

   ☒ claims Nos. 1-28, with regard to industrial applicability

   because:

   ☒ the said international application, or the said claims Nos. 1-28, with regard to industrial applicability relate to the following subject matter which does not require an international preliminary examination (specify):

   see separate sheet

   ☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos.

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the Standard.

   ☐ the computer readable form has not been furnished or does not comply with the Standard.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees, the applicant has:

   ☒ restricted the claims.

   ☐ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

   ☐ complied with.

   ☐ not complied with for the following reasons:

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☐ all parts.

☒ the parts relating to claims Nos. 1-28,35 .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-20,22-28
	No:	Claims	21,35
Inventive step (IS)	Yes:	Claims	-
	No:	Claims	1-28,35
Industrial applicability (IA)	Yes:	Claims	35
	No:	Claims	

2. Citations and explanations

see separate sheet

Re Item III
Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claims 1-28 relate to human embryos and their scope. For the assessment of patentability of subject-matter relating to human embryos, no unified criteria exist in the PCT Contracting States. The EPO, for example, considers subject-matter relating to human embryos to be contrary to morality. Therefore, claims 1-28 are not allowable before the EPO.
For the assessment of the present claims 1-28 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item V
Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

The following documents are referred to in this communication:

D1: US-B1-6 200 806 (THOMSON JAMES A) 13 March 2001
D2: ROPETER-SCHARFENSTEIN M ET AL,, JOURNAL OF ANIMAL BREEDING AND GENETICS, vol. 113, pages 427-436, 1996
D3: WO 99 27076 A (LORING JEANNE F ;ARC GENOMIC RESEARCH (US)) 3 June 1999
D4: US-A-5 905 042 (GOLUEKE PAUL J ET AL) 18 May 1999
D5: US-B1-6 190 910 (KAMON TOSHIO ET AL) 20 February 2001

1.1) The subject-matter of claim 35 is not new (Article 33(2) PCT)
because D1 discloses a kit for a method for obtaining a human BS cell comprising, in separate compartments, mouse embryonic feeder cells and pronase and further comprising blastocysts with an intact zona pellucida (D1: col. 8, l. 25-43).

1.2) The subject-matter of claim 21 relates to a product being defined in terms of

processes for its preparation (product-by-process' claims). A product, however, is not rendered novel merely by the fact that it is produced by means of a new process . According to the description of the present application, the advantage of the method of claim 1 lies in a simpler, faster and non-traumatic procedure which results in a higher output of stem cell lines (page 5, lines 13-21). This means that the method of claim 1 allegedly results in more but still the same product as the method of D1. Consequently, the disclosures made by D1 anticipate the novelty of claim 21 (Article 33(2) PCT) because D1 has already described the use of a human BS cell line for the preparation of differentiated cells (D1: col. 6, l. 35-45).

2) The subject-matter of claims 1-28 does not involve an inventive step (Article 33(3) PCT) for the following reason:

i) Document D1 is considered to represent the most relevant state of the art because it discloses a method for obtaining a pluripotent human BS cell line from which the subject-matter of claim 1 differs in that, instead of immunosurgery, mechanical dissection is used to isolate the inner cell mass (ICM) cells (D1: col. 8, l. 14 - col. 9, l. 32).

ii) The problem to be solved by the present invention may therefore be regarded as the improvement of the method of D1 with reduced risk of damaging the ICM cells during their isolation.

iii) The solution proposed in claim 1 of the present application, i.e. the mechanical dissection cannot be considered as involving an inventive step because D2 has already related immunosurgery with a potential risk of inflicting damage to the ICM cells and proposed a mechanical approach instead (D2: page 431, penultimate paragraph).

iv) D2 relates to porcine ICM cells. However, D2 is not the only document that relates to the mechanical dissection of ICM cells. Other documents have already demonstrated its applicability in other species: rat (D3), ungulates (D4) and mouse (D5). Consequently, it was already known to the skilled person that the teachings from D2 do not only apply to porcine blastocysts.
It would therefore be obvious to the person skilled in the art, to employ mechanical dissection with corresponding effect in the method of D1 for obtaining human BS cells,

thus arriving at the method of claim 1.

v) Claims 2-28 do not appear to contain any additional features which, in combination with the features of any claim to which they refer, relate to an inventive step, because they do not contain any features that were not already described in either D1 or D2.

3) Claims 22-28 are not clear (Article 6 PCT) because the matter for which protection is sought is not defined. The claims attempt to define the subject-matter in terms of the result to be achieved. In fact, said claims 22-28 appear to relate to the same subject-matter, i.e. they are redundant: a pluripotent cell, per definition, has the ability to differentiate into many types of specialized cells like insulin-producing cells wherein such differentiation will not depend on the pluripotent cell but rather on the cultivation media used.

Claims, PCT/EP02/14895.

1. A method for obtaining a pluripotent human blastocyst-derived stem cell line, the method comprising the steps of

i) using a fertilized oocyte, having a grade 1 or 2, to obtain a blastocyst, having a grade A or B,

ii) co-culturing the blastocyst with feeder cells for establishing one or more colonies of inner cell mass cells,

iii) isolating the inner cell mass cells by mechanical dissection,

iv) co-culturing of the inner cell mass cells with feeder cells to obtain a blastocyst-derived stem cell line.

v) optionally, propagation of the blastocyst-derived stem cell line.

2. A method for obtaining a pluripotent human blastocyst-derived stem cell line, the method comprising the steps of

i) using a fertilized oocyte having a grade 1 or 2, to obtain a blastocyst, optionally having a grade A or B,

ii) co-culturing the blastocyst with feeder cells for establishing one or more colonies of inner cell mass cells,

iii) isolating the inner cell mass cells by mechanical dissection,

iv) co-culturing of the inner cell mass cells with feeder cells to obtain a blastocyst-derived stem cell line.

v) optionally, propagation of the blastocyst-derived stem cell line.

3. A method for obtaining a pluripotent human blastocyst-derived stem cell line, the method comprising the steps of

i) using a fertilized oocyte optionally, having a grade 1 or 2, to obtain a blastocyst, having a grade A or B,

ii) co-culturing the blastocyst with feeder cells for establishing one or more colonies of inner cell mass cells,

iii) isolating the inner cell mass cells by mechanical dissection,

iv) co-culturing of the inner cell mass cells with feeder cells to obtain a blastocyst-derived stem cell line.

v) optionally, propagation of the blastocyst-derived stem cell line.

4. A method for obtaining a pluripotent human blastocyst-derived stem cell line, the method comprising the steps of

i) using a fertilized oocyte optionally, having a grade 1 or 2, to obtain a blastocyst, optionally having a grade A or B,

ii) co-culturing the blastocyst with feeder cells for establishing one or more colonies of inner cell mass cells,

iii) isolating the inner cell mass cells by mechanical dissection,

iv) co-culturing of the inner cell mass cells with feeder cells to obtain a blastocyst-derived stem cell line,

v) propagation of the blastocyst-derived stem cell line culturing the stem cells with feeder cells of a density of less than about 60,000 cells per cm^2, such as e.g. less than about 55,000 cells per cm^2, or less than about 50,000 cells per cm^2, such as about 45,000 cells per cm^2.

5. A method according to any of the claims 1-4 in which the blastocyst in step i) is a spontaneously hatched blastocyst.

6. A method according to any of the claims 1-5 in which the blastocyst-derived stem cell line is stable.

7. A method according to any of the claims 1-6 wherein the blastocyst-derived stem cell line is propagated.

8. A method according to claim 7, in which the propagation of blastocyst-derived stem cell line comprises passage of the stem cell line every 4-5 days.

9. A method according to claims 7-8, in which the propagation of blastocyst-derived stem cell line comprises culturing the stem cells with feeder cells of a density of less than about 60,000 cells per cm^2, such as e.g. less than about 55,000 cells per cm^2, or less than about 50,000 cells per cm^2.

10. A method according to claim 9, in which the propagation of blastocyst-derived stem cell line comprises culturing the stem cells with feeder cells of a density of about 45,000 cells per cm^2.

11. A method according to claims 7-10, in which the propagation of blastocyst-derived stem cell line comprises passage of the feeder cells at the most 3 times, such as e.g. at the most 2 times.

12. A method according to any of the claims 1-11 in which the zona pellucida of the blastocyst has been at least partially digested prior to step ii).

13. A method according to claim 12 in which the zona pellucida of the blastocyst has been at least partially digested with a digestive agent selected from the group comprising acidic reacting substances, enzymes and mixtures thereof.

14. A method according to any of the claims 1-13 in which step ii) and/or step iv) is performed in an agent that improves the attachment of the blastocysts and/or if relevant the inner cell mass cells to the feeder cells.

15. A method according to claim 14 wherein the agent is a hyaluronic acid.

16. A method according to any of the claims 1-15 in which the feeder cells are embryonic feeder cells.

17. A method according to any of the claims 1-16 in which the feeder cells employed in steps ii) and iv) are the same or different and originate from animal source.

18. A method according to claim 17 wherein the feeder cells are of mouse or human origin.

19. A method according to any of the claims 1-18, wherein the feeder cells are mitotically inactivated.

20. A method according to any of the claims 1-19, wherein the stem cell line
i) exhibits proliferation capacity in an undifferentiated state for more than 21 months when grown on mitotically inactivated embryonic feeder cells, and
ii) exhibits normal euploid chromosomal karyotype, and
iii) maintains potential to develop into derivatives of all types of germ layers both *in vitro* and *in vivo*, and

iv) exhibits at least two of the following molecular markers OCT-4, alkaline phosphatase, the carbohydrate epitopes SSEA-3, SSEA-4, TRA 1-60, TRA 1-81, and the protein core of a keratin sulfate/chondroitin sulfate pericellular matrix proteinglycan recognized by the monoclonal antibody GCTM-2, and

v) does not exhibit molecular marker SSEA-1 or other differentiation markers, and

vi) retains its pluripotency and forms teratomas in vivo when injected into immuno-compromised mice, and

vii) is capable of differentiate.

21. Use of the human blastocyst-derived stem cell line obtained by the method according to any of the claims 1-20 for the preparation of differentiated cells.

22. A method according to any of the claims 1-20, wherein the stem cell line has the ability of differentiating into an insulin producing cells.

23. A method according to claim 22, wherein the insulin producing cells are capable of forming islet-like structures.

24. A method according to claims 22 or 23, wherein the amount of insulin producing β-cells which are derived from the pluripotent human BS cell line is higher than 25%, such as e.g. higher than 35%, or higher than 40%, or higher than 45%, or higher than 50%.

25. A method according to claims 22-24, wherein the insulin producing cell line produces at least about 300 ng insulin/mg total protein such as at least about 380 ng insulin/mg total protein or at least about 450 ng insulin/mg total protein.

26. A method according to any of the claims 1-20 or 22-25, wherein the blastocyst-derived stem cells have the ability to differentiate into differentiated cells, which display the expression of pancreatic cell type markers, including at least one of insulin, Glut-2, Pdx-1, glucokinase, glucagon and somatostatin.

27. A method according to any of the claims 1-20 or 22-26, wherein the blastocyst-derived stem cells have the ability to differentiate into insulin-producing cells characterized by their organization into islet-like structures comprising an inner core of β-cells surrounded by an outer layer of neuron-type cells, which neuron-type cells

display expression of at least one of the following neuronal cell type markers, including neuron-specific β-III tubulin (TUJ1), NeuN, DoubleCortin, tyrosine hydroxylase and Map 2.

28. A method according to any of the claims 1-20, wherein the blastocyst-derived stem cells are capable of being made into differentiated cells, which display the expression of at least one of the following neuronal cell type markers, including neuron-specific β-III tubulin (TUJ1), NeuN, DoubleCortin, tyrosine hydroxylase and Map 2.

29. Use of a preparation of differentiated cells derived from the blastocyst-derived stem cells obtained by the method according to any of the claims 1-20 or 22-28 for the manufacture of a medicament for the prevention or treatment of pathologies or diseases caused by tissue degeneration.

30. Use of a preparation of differentiated cells derived from the blastocyst-derived stem cells obtained by the method according to any of the claims 1-20 or 22-27 for the manufacture of a medicament for the prevention or treatment of pathologies or diseases in the pancreas.

31. Use according to claim 30, in which the disease is diabetes.

32. Use according to claim 28 or 29, in which the disease is type 1 diabetes.

33. Use of a preparation of differentiated cells derived from the blastocyst-derived stem cell line obtained by the method according to any of the claims 1-20 or 28 for the manufacture of a medicament for the prevention or treatment of pathologies or diseases in the nervous system.

34. Use according to claim 33, in which the disease is selected from the group consisting of multiple schlerosis, spinal chord injury, encephalopathies, Parkinson's disease, Huntingdon's disease, stroke, traumatic brain injuries, hypoxia induced brain injuries, ischemia induced brain injuries, hypoglycemic brain injuries, degenerative disorders of the nervous system, brain tumors and neuropathies in the peripheral nervous system.

35. A kit for performing the method according to any of the claims 1-20, comprising human blastocysts with an intact zona pellucida or spontaneously hatched blastocysts and at least two of the following components in separate compartments; hyaluronic acid, pronase, BS-cell medium, and human or mouse embryonic feeder cells.

36. A method for producing an essentially pure preparation of insulin-producing differentiated stem cells, comprising the steps of;

i) expanding human blastocyst-derived stem cells by growing these on an inactivated feeder cell layer in a suitable medium;

ii) generating blastocyst-derived stem cell bodies by dissociating colonies formed in step i) into smaller aggregates or individual cells, followed by transferring said aggregates or individual cells to non-adherent containers where they are incubated in a suitable medium;

iii) plating the blastocyst-derived stem cell bodies in containers in a suitable medium;

iv) selecting nestin-positive neural precursors in ITFSn medium;

v) expanding pancreatic endocrine progenitor cells in, N2-medium comprising B27 media complement and basic fibroblast growth factor;

vi) changing the medium to a basic fibroblast growth factor-free N2 medium.

37. A method according to claim 36 in which the human blastocyst-derived stem cells are obtained by the method according to any of the claims 1-20.

38. A method according to claims 36-37 in which the medium used in step i) is human blastocyst-derived stem cell medium.

39. A method according to claim 36-38 in which the medium used in step ii) is blastocyst-derived stem cell body medium.

40. A method according to claims 36-39 in which the medium used in step iii) is blastocyst-derived stem cell body medium.

41. A method according to claims 36-40 in which nicotinamide is added after step vi).

42. An essentially pure preparation of differentiated stem cells, wherein the cells display the expression of pancreatic cell type markers, including at least one of insulin, Glut-2, Pdx-1, glucokinase, glucagon and somatostatin.

43. The preparation according to claim 42, which is capable of producing at least about 320 ng insulin/mg total protein such as at least about 380 ng insulin/mg total protein or at least about 420 ng insulin/mg total protein.

44. The preparation according to claims 42 or 43, in which preparation the proportion of insulin producing cells is at least 25%, such as e.g. at least 35%, or at least 45%, or at least 50%.

45. The preparation according to claims 42-44, characterized by its organization into islet-like structures comprising an inner core of β-cells surrounded by an outer layer of neuron-type cells, which neuron-type cells display expression of at least one of the following neuronal cell type markers, including neuron-specific β-III tubulin (TUJ1), NeuN, DoubleCortin, tyrosine hydroxylase and Map 2.

46. The preparation according to claims 42-45, obtained by the method according to claims 36-41.

47. An essentially pure preparation of differentiated stem cells, wherein the cells display the expression of at least one of the following neuronal cell type markers, including neuron-specific β-III tubulin (TUJ1), NeuN, DoubleCortin, tyrosine hydroxylase and Map 2.

48. The preparation according to claim 47, obtained by the method according to claims 36-41.

49. An essentially pure preparation of cells obtainable by the method according to claims 36-41.

50. Use of a preparation according to claims 42-46 for the manufacture of a medicament for the prevention or treatment of pathologies or diseases in the pancreas.

51. Use according to claim 50, in which the disease is diabetes.

52. Use according to claim 50 or 51, in which the disease is type 1 diabetes.

53. Use of a preparation according to claims 47-48 for the manufacture of a medicament for the treatment of pathologies or diseases in the nervous system.

54. Use according to claim 53, in which the disease is selected from the group consisting of multiple schlerosis, spinal chord injury, encephalopathies, Parkinson's disease, Huntingdon's disease, stroke, traumatic brain injuries, hypoxia induced brain injuries, ischemia induced brain injuries, hypoglycemic brain injuries, degenerative disorders of the nervous system, brain tumors and neuropathies in the peripheral nervous system.

55. Kit for performing the method according to claims 36-41, comprising at least two of the following components in separate compartments; mitomycin C, hBS medium, BS cell body medium, ITSFn-medium, N2-medium, B27-media supplement, nicotinamide, and bFGF.

56. Kit according to claim 55, further comprising an essentially pure human blastocyst-derinved stem cell line obtained by the method according to any of the claims 1-20.